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APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANT: Arnold L. Demain and Aiqi Fang

TITLE: Clostridium Difficile Culture and Toxin Production Methods

CLOSTRIDIUM DIFFICILE CULTURE AND TOXIN PRODUCTION METHODS

Cross-Reference to Related Applications

This application claims priority from U.S. Provisional Application No. 60/436,378, filed December 23, 2002.

Background of the Invention

This invention relates to methods for culturing *Clostridium difficile* and producing *Clostridium difficile* toxins.

Clostridium difficile is a gram-positive, spore-forming, toxigenic bacterium that causes antibiotic-associated diarrhea, which can progress into severe and sometimes fatal colitis. These conditions can occur when the normal intestinal flora is disrupted by, e.g., antibiotic or anti-neoplastic therapy. Such disruption enables *C. difficile* to become established in the colon, where it produces the causative agents of these conditions: two high molecular weight toxins, Toxin A and Toxin B. Both of these polypeptides are cytotoxins, but Toxin B is greater than 1,000-fold more potent than Toxin A. Toxin A is also an enterotoxin, as it causes accumulation of fluid in ligated animal intestinal loops.

C. difficile Toxins A and B are encoded by two separate but closely linked genes that together form part of a 19.6 kilobase region known as the "toxigenic element" or the "pathogenicity locus." The Toxin A and B genes and proteins are highly homologous, as it is likely that the genes evolved by duplication. Toxins A and B are produced simultaneously in *C. difficile* strain VPI 10463 (ATCC 43255), and the ratio of the produced toxins is usually 3:1, respectively (Karlsson et al., Microbiology 145:1683-1693, 1999). The toxins begin to be formed during the exponential growth phase, and they are usually released from the bacteria between 36 and 72 hours of culture. Toxins present within the bacteria can be released earlier by sonication or by use of a French pressure cell.

Media for the growth of *C. difficile* typically contain animal and dairy by-products as sources of proteins, amino acids, and other nutrients required for growth (see, e.g., Holbrook et al., J. Appl. Bacteriol. 42:259-273, 1977). Manufacturers of such media have used complex ingredients, such as casein digests and meat extracts, to maximize toxin production.

Summary of the Invention

The invention provides methods of culturing *C. difficile*, which involve growing the *C. difficile* in media that are substantially free of animal-derived products (e.g., media that lack animal-derived products). These media can include one or more compounds derived from a vegetable (e.g., a soybean), such as hydrolyzed soy. These media can also, optionally, include an iron source. The culturing can, optionally, be carried out under anaerobic conditions.

The methods of the invention can be used to grow *C. difficile* in seed cultures, for example, seed cultures that are started by inoculation from a stock culture that was grown in medium that was substantially free of animal-derived products. The methods can also be used to grow *C. difficile* in fermentation cultures, which can have been inoculated from seed cultures (e.g., first or second seed cultures) that were grown in medium that was substantially free of animal-derived products. These methods can further include isolating *C. difficile* toxins (i.e., Toxin A and/or Toxin B) from the medium.

The invention also provides methods for obtaining *C. difficile* toxins. These methods involve (i) culturing *C. difficile* in a first medium that is substantially free of animal-derived products, under conditions that facilitate growth of *C. difficile*; (ii) inoculating a second medium that is substantially free of animal-derived products with all or a portion of the first medium after the culturing; (iii) culturing the inoculated second medium under conditions that facilitate growth of *C. difficile* and toxin production; and (iv) isolating *C. difficile* toxins from the second medium.

The media used in these methods can include one or more compounds that are derived from a vegetable (e.g., a soybean), such as hydrolyzed soy. Any or all of the culturing steps of these methods can, optionally, be carried out under anaerobic conditions. Moreover, culturing in the first medium can be started by inoculation with a previous *C. difficile* culture (e.g., a stock culture or a previous seed culture) that was cultured in medium that was substantially free of animal-derived products.

Also included in the invention are compositions that include a culture medium that is substantially free of animal products and also contain *Clostridium difficile*. These compositions can also include one or more compounds that are derived from a vegetable (e.g., a soybean), such as hydrolyzed soy. Optionally, these compositions can also include an iron source.

The invention provides several advantages. For example, use of animal product-free media provides an important safeguard against the possibility of contamination of medical products (e.g., vaccines) that are derived from the cultured bacteria with undesirable material. Such contaminants include, for example, the causative agent of Bovine Spongoform Encephalopathy (i.e., mad cow disease or BSE), antigenic peptides that stimulate undesired immune reactions in immunized subjects (e.g., anaphylactic reactions), and viruses. The invention is also advantageous because it facilitates high efficiency bacterial growth and toxin production. Other features and advantages of the invention will be apparent from the following detailed description and the claims.

Detailed Description

The invention provides methods and compositions for use in growing *Clostridium difficile* and producing the *C. difficile* toxins, toxins A and B. These toxins can be used, for example, in vaccination methods or in the preparation of toxoids, which can in turn be used in vaccines. As is discussed further below, the methods and compositions of the invention employ culture media that contain significantly reduced levels of animal products, such as meat or dairy by-products, if any. The invention is based on the present inventors' discovery that animal-based products, which traditionally have been used in media for culturing *C. difficile*, are not required to achieve efficient culture of these bacteria. As is described in further detail below, the inventors found that vegetable-based products can replace animal-based products in these media, leading to high levels of bacterial growth and toxin production. As is noted above, replacing the animal components of culture media with vegetable-based products reduces the potential for contamination of medical products made from the bacteria (e.g., toxins, toxoids, and cell preparations) with undesirable molecules, such as certain proteins and viruses that may exist in animals.

The vegetable-based products in the media used in the present invention can be, for example, soy-based products. The soy-based products can be, optionally, hydrolyzed and, preferably, are soluble in water. However, insoluble soy products can also be used in the methods of the invention. Common animal products that can be substituted by soy products include beef heart infusion (BHI), peptones (e.g., tryptones), and dairy by-products, such as

animal milk, or casein or its hydrosylates. Examples of soy products that can be used in the invention, and their sources, include:

Tekniscience: Soy Peptone A1, Soy Peptone A2, Soy Peptone A3, Plant Peptone E1, Plant Peptone ET1, and Wheat Peptone E1;

Quest: HY-Soy, HY-Soy T, AMI-Soy, NZ-Soy, NZ-Soy BL4, and NZ-Soy BL7;

DMV: SE50M, SE70M, SE50MK, WGE80BT, WGE80M, CNE50M, and SE70BT;

Marcor: Soy Peptone Type AB, Soy Peptone Type AC, Soy Peptone Type SL, Soy Peptone Type II, and Soy Peptone Type F;

Oxoid: Vegetable Peptone and Vegetable Peptone No. 1;

Gibco: Soy Peptone; and

Difco: Bacsoytone.

Media containing vegetable (e.g., soy) products for the growth of *C. difficile* can be similar to commonly used growth media containing animal derived products (e.g., TY or TYM media; see below), except that all or substantially all of the animal-derived products are replaced with the vegetable-derived products. In addition, ingredients in TY, TYM, and similar media that are not essential for growth of *C. difficile* in media containing soy-based products can also be included in the media nonetheless, to enhance growth and toxin production.

Generally, growth of *C. difficile* according to the methods of the invention proceeds in at least two phases: seed growth and fermentation. A relatively small seed culture is first grown by inoculation from a stock culture, e.g., a working cell bank, and this seed is used either to inoculate a second seed culture or to inoculate a relatively large fermentation culture. As is well understood in the art, the number of seed cultures used depends, for example, on the size and volume of the fermentation step. As is described further below, the fermentation media used in the methods of the invention lacks animal products, as described herein. Preferably, the seed and stock cultures employ media that lack such products as well, although this is not absolutely required.

Generally, the culturing stages of the methods of the invention (both seed and fermentation) are carried out under anaerobic conditions, although aerobic conditions for either of these phases may be used as well. Approaches to anaerobic culture of bacteria, such as *C. difficile*, are well known in the art and can employ, for example, nitrogen gas or a mixture of nitrogen and hydrogen gases. The gas can either be bubbled through the medium during

fermentation or passed through the area above the liquid in a culture chamber (i.e., the chamber headspace). Preferably, the nitrogen gas or nitrogen/hydrogen gas mixture is passed through the headspace in a continuous manner.

The seed growth phase (or phases) are generally carried out to scale-up the quantity of the microorganism from a stored culture, so that it can be used as an inoculant for the fermentation phase. The seed growth phase can also be carried out to allow relatively dormant microbes in stored cultures to become rejuvenated and to grow into actively growing cultures. Further, the volume and quantity of viable microorganisms used to inoculate the fermentation culture can be controlled more accurately if taken from an actively growing culture (i.e., a seed culture), rather than if taken from a stored culture. In addition, as is noted above, more than one (e.g., two or three) seed growth phases can be used to scale-up the quantity of *C. difficile* for inoculation of the fermentation medium. Alternatively, growth of *C. difficile* in the fermentation phase can proceed directly from the stored culture by direct inoculation, if desired.

To start the fermentation phase, a portion or all of a seed culture containing *C. difficile* is used to inoculate fermentation medium. Fermentation is used to produce the maximum amount of the bacterium in a large-scale anaerobic environment (Ljungdahl et al., "Manual of Industrial Microbiology and Biotechnology," 1986, ed., Demain and Solomon, American Society for Microbiology, Washington, D.C., p. 84).

C. difficile toxins can be isolated and purified from fermentation cultures using well known protein purification methods. (See, e.g., Coligan et al., "Current Protocols in Protein Science," Wiley & Sons; Ozutsumi et al., Appl. Environ. Microbiol. 49:939-943, 1985; and Kim et al., Infection and Immunity 55:2984-2992, 1987; which are incorporated herein by reference.) The purified toxins can then, for example, be inactivated by formaldehyde treatment, so that they can be used, e.g., in immunization methods (see, e.g., Libby et al., Infection and Immunity 36:822-829, 1982).

Additional details concerning the methods of the invention are provided as follows.

Seed Culture

As is discussed above, cultures of *C. difficile* can be grown in one or more seed cultures to produce a sufficient quantity of active cultures for the inoculation of fermentation medium. Also as is discussed above, the number of steps involving growth in a seed medium (e.g.,

inoculation of a second seed medium by a first seed culture) can vary, depending on the scale of the production in the fermentation phase. An example of an animal-based seed medium that has been used to culture *C. difficile* is TYM, which includes tryptone peptone, yeast extract, mannitol, and glycerol (see below). This medium can be adapted for use in the present invention by replacing the tryptone peptone with a vegetable-derived product, such as a soy-based product. For example, a hydrolyzed soy product, which may be soluble in water, can be used. Any source of such soy-based products may be used in the present invention including, for example, those listed above (e.g., NZ Soy BL4 or Soy Peptone A3).

Concentrations of the soy product in the seed medium can range, for example, between 5 and 200 g/L, e.g., 20-150 g/L, 25-100 g/L, or 50-75 g/L. Concentrations of a carbon source (e.g., glucose, mannitol, or glycerol) in this medium can range, for example, between 0.1 g/L and 20.0 g/L, e.g., 0.5-10.0 g/L or 1-5 g/L. Any combination of carbon sources can be used in the medium. For example, as with TYM medium, mannitol and glycerol can both be included.

To enhance toxin A production, an iron compound, such as, for example, reduced iron powder (e.g., 0.1-5.0 g/L, 0.25-3.0 g/L, or 0.5-1.5 g/L), $FeSO_4 \cdot 7H_2O$ (e.g., 1-100 mg/L or 40-60 mg/L), or ferrous gluconate (e.g., 50-400 mg/L, 150-300 mg/L, or 200-250 mg/L), can be included in the seed culture media. Additional examples of iron sources that can be used in the invention include non-reduced iron powder (J.T. Baker and Sigma-Aldrich), iron wire (e.g., Puratronic, Alfo Aesoar A. Johnson Matthey Co., and Sigma-Aldrich), iron foil, ferric citrate, and ferrous ammonium sulfate. When iron powder is used, it can be autoclaved together with other ingredients of the fermentation medium. When iron wire is used, it can have a diameter of, for example, between approximately 0.05 mm and 2.0 mm, e.g., a diameter of 0.075 mm (e.g., Puratronic; 99.995% metal basis pure). The preferred pH level of the seed medium prior to growth can range between 6.8 and 8.5, and thus can be, for example, approximately 6.8 or 7.5.

As is noted above, growth of *C. difficile* in the seed medium may proceed in one or more stages, for example, in two stages. In stage one, a culture of *C. difficile* is suspended in seed medium and is incubated at a temperature between 30-40°C, preferably 34±1°C, for 24-48 hours in an anaerobic environment. In stage two, a portion or all of the stage one seed medium containing *C. difficile* is used to inoculate a stage two seed medium for further growth. After inoculation, the stage two medium is incubated at a temperature between 30-40°C, preferably at 34±1°C or 37±1°C, for approximately 1-4 days, e.g., for 1-2 days, also in an anaerobic

environment. Preferably, growth in seed media at any stage does not result in cell lysis before inoculation of fermentation media. Additional growth in a third (or further) stage seed medium can be carried out as well, if desired. An appropriate concentration of seed culture to use to inoculate fermentation media can be determined by those of skill in this art and can range, for example, from 0.1-10%. As specific examples, concentrations of 0.5, 1.0, or 5.0% can be used.

Fermentation Culture

An example of an animal-based fermentation medium that has been used to culture *C. difficile* is TY, which includes tryptone peptone, yeast extract, and sodium thioglycolate (see below). This medium can be adapted for use in the present invention by replacing the tryptone peptone with a vegetable-derived product, such as a soy-based product. For example, a hydrolyzed soy product, which preferably is soluble in water, can be used. Any source of such soy-based products can be used including, for example, those listed above (e.g., NZ-Soy BL7, NZ-Soy BL4, NZ Soy, Oxoid Vegetable Peptone No. 1, or WGE80M).

The concentration of soy product in the fermentation medium can range between 5 and 200 g/L, 20-150 g/L, 25-100 g/L, or 50-75 g/L. Optionally, the medium can include an iron source, such as those listed above, in the amounts listed above. The pH of the fermentation medium can range between 7.0 and 8.5. Thus, for example, the pH can be 6.8 or 7.5.

Fermentation can be carried out in an anaerobic chamber at approximately 34±1°C or 37±1°C for approximately 4 to 9 days. Growth can be monitored by measuring the optical density (O.D.) of the medium. Fermentation can be stopped after cell lysis has proceeded for at least 48 hours, as determined by growth measurement (optical density). As cells lyse, the O.D. of the medium will decrease.

C. difficile can be cultivated by fermentation with continuous exposure to a 90% nitrogen/10% hydrogen mixture or to 100% nitrogen. Nitrogen gas or a mixture of nitrogen and hydrogen gas may also be bubbled through the medium during fermentation. In addition, agitation (approximately 100 rpm) of the culture during fermentation can be used. General methods of fermentation for *C. difficile* are well known to those skilled in the art, and can be used in the invention.

Stock Culture

As is noted above, the media in which stock cultures (i.e., working cell bank cultures) for inoculating seed cultures is present can include, optionally, vegetable products in place of animal products as described herein. For example, the media can include a soy product in place of tryptone peptone in TYM medium (see below). The soy product can be any of those listed above, e.g., Soy Peptone A3 or NZ-Soy BL4.

According to another alternative, cultures of *C. difficile* used for long-term storage and for inoculation of seed media can be grown and lyophilized in soy-milk prior to storage at 4°C. However, to maintain media that are substantially free of animal by-products throughout the production of *C. difficile* toxins, it is preferred that the initial culture of *C. difficile* be preserved in soy milk, and not animal milk. The stored culture, which can be lyophilized, is thus produced by growth in media containing proteins derived from soy and lacking animal by-products. Growth of *C. difficile* in fermentation medium can proceed by inoculation directly from such a stored, lyophilized culture, or through seed cultures, as is discussed above.

The invention is based, in part, on the experimental results that are described below.

Experimental Results

Materials and Methods

1. Seed:

i) Tryptone-Yeast extract-Mannitol Medium (TYM, g/L)

Tryptone peptone (Difco)	24
Yeast extract	12
Mannitol	10
Glycerol	1
pH	6.8

ii) Preparation of seed medium:

Tryptone peptone (Difco) 2.4 g, yeast extract 1.2 g, mannitol 1 g and glycerol 0.1 g were added into 100 ml d.d. water. pH was adjusted to 6.8 with 5N NaOH.

10 ml seed medium was dispensed into each seed tube (16 x 150 mm) and 40 ml seed medium into each 2510-DeLong Bellco Culture Flask (125 ml).

Autoclaving was done at 121°C for 30 minutes, and the vessels were then immediately moved to a COY Anaerobic Chamber (Coy Laboratory Products Inc., Grass lake, MI) filled with 10% carbon dioxide (CO_2) plus 10% hydrogen (H_2) and 80% nitrogen (N_2).

iii) Seed culture:

First stage seed culture: A vial of working cell bank (WCB) culture (1 ml) was transferred into a 16 x 150 mm test tube containing 10 ml seed medium (TYM) and incubated at 35±1°C for 24 hours.

Second stage seed culture: 1 ml first stage seed culture was added as an inoculum to a 125 ml DeLong Bellco Culture Flask containing 40 ml seed medium (TYM). The flasks were incubated at 37±1°C for 24 hours.

2. Fermentation

i) Fermentation media:

A. Tryptone-Yeast extract-Mannitol Medium (TYM, g/L)

Tryptone peptone (Difco)	24
Yeast extract	12
Mannitol	10
Glycerol	1
pH	6.8

B. TY medium (g/L):

Tryptone peptone (Difco)	30
Yeast extract	20
Sodium thioglycolate	1
pH	6.8

C. TYG medium (g/L):

Tryptone peptone (Difco)	30
Yeast extract	20
Glucose	10
Sodium thioglycolate	1
pH	6.8

D. Tryptone-Yeast extract-Mannitol Medium (TYM-2, g/L)

Tryptone peptone (Difco)	12
Yeast extract	24
Mannitol	10
Glycerol	5
pH	8.0 (adjust pH with KOH)

ii) Non-animal peptones:

Tekniscience: Soy Peptone A1, Soy Peptone A2, Soy Peptone A3, Plant Peptone E1, Plant Peptone ET1, and Wheat Peptone E1;

Quest: HY-Soy, HY-Soy T, AMI-Soy, NZ-Soy, NZ-Soy BL4, and NZ-Soy BL7;

DMV: SE50M, SE70M, SE50MK, WGE80BT, WGE80M, CNE50M, and SE70BT;

Marcor: Soy Peptone Type AB, Soy Peptone Type AC, Soy Peptone Type SL, Soy Peptone Type II, and Soy Peptone Type F;

Oxoid: Vegetable Peptone and Vegetable Peptone No. 1.

iii) Preparation of fermentation media:

At first, 3 g peptones were individually placed into 150 ml bottles. The fermentation medium was prepared without the peptones and 100 ml medium was added to each bottle, dissolved with a magnetic stirring bar, and then the pH was adjusted to 6.8 with 3N HCl or 5N NaOH. Autoclaving was done at 121°C for 30 minutes, then the bottles were immediately moved to a COY Anaerobic Chamber filled with 10% carbon dioxide (CO_2) plus 10% hydrogen (H_2) and 80% nitrogen (N_2). Eight ml medium was added to each 16 x 100 mm test tube.

iv) Cultivation:

40 µl seed culture (step 2) was used as an inoculum (0.5%) for each 8 ml of production medium in 16 x 100 mm test tubes. Three tubes were used for each variable. The tubes were incubated in the anaerobic chamber at 37±1°C for 5 days. Growth (OD) was measured before mixing and after mixing (excepting the case of insoluble peptones) with a Turner Spectrophotometer (Model 330) at 540 nm after 24 hours after inoculation. One uninoculated tube was used as a blank to zero in the spectrophotometer. The cultivation was usually stopped on the 3rd and the 5th day.

3. Toxin production was measured by the ELISA method.

A Fusion Universal Microplate Analyzer (Packard, Meriden, CT) was used for reading of the ELISA plates using filters 405 nm and 490 nm.

4. Microorganism: *Clostridium difficile* VPI10463

Results

Section 1: To compare different complex fermentation media for Toxin A production.

Table 1. Cell growth and Toxin A production in the different fermentation media: TYM medium (control), TY medium, and TY medium +10 g/L glucose.

Media	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 5 days	7 days
TYM	0.97	27	47
TY	0.95	2500	2420
TYG	1.05	26	67

Table 1 shows that Toxin A production was best in TY medium, which contains 1 g/L sodium thioglycolate. Glucose slightly increased cell growth, but markedly inhibited Toxin A production.

Table 2. Cell growth and Toxin A production in TYM-2 fermentation medium.

Media	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
TYM-2	1.20	<160	<160
TY	0.79	6818	8416

Table 2 shows that cell growth was increased, but Toxin A production was markedly inhibited, in TYM-2 fermentation medium, which is similar to TYM, but contains higher levels of glycerol and yeast extract and a lower level of Tryptone.

Tables 1 and 2 show that TY is a superior fermentation medium when using Tryptone as a nitrogen source.

Section 2: To determine the effect of carbon sources in the fermentation medium on cell growth and Toxin A production.

Table 3. The effect of mannitol and glycerol as carbon sources on cell growth and Toxin A production in the fermentation medium with Hy-Soy as replacement for Tryptone.

Carbon sources (g/L)	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
Without carbon source	0.66	3700	3820
Glucose (10)	0.90	218	244
Mannitol (10)	0.84	158	78
Glycerol (1)	0.68	3650	3820
Mannitol (10) + Glycerol (1)	0.81	140	78

Table 3 shows that glucose and mannitol markedly inhibited Toxin A production, but glycerol did not.

Section 3: To determine whether non-animal peptone products can replace Tryptone in fermentation medium for cell growth and Toxin A production

Table 4. Comparison of different non-animal peptones as replacements for Tryptone in TYM fermentation medium.

Peptone	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 5 days	7 days
Tryptone	0.97	27	47
Hy-Soy	0.76	66	80
SE50MK	0.59	<6	<6
Soy peptone A1	0.78	<6	<6
Soy peptone A2	0.86	25	24
Soy peptone A3	0.91	29	28
Plant peptone E1	0.82	64	34
Plant peptone ET1*	--	<6	<6
Wheat peptone E1	0.72	<6	<6

*The peptone is insoluble.

Table 4 shows that Hy-Soy was the best choice of 8 different non-animal peptones in TYM fermentation medium as a Tryptone replacement for Toxin A production. However, TYM is not the medium of choice for fermentation. Thus, we continued our examination of peptones in TY medium, less Tryptone.

Table 5. Comparison of different non-animal peptones as replacements for Tryptone in TY fermentation medium.

Peptone	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
Tryptone	0.88	1700	4250
Hy-Soy	0.66	3700	3820
Plant peptone E1*	--	2700	4000
Vegetable Peptone	1.07	218	288
Vegetable Peptone No. 1	0.75	2580	10000
Soy Peptone Type II	0.64	4200	4250
Soy Peptone Type AC	0.79	3650	3475
Soy Peptone Type AB	0.77	4000	3110
Soy Peptone Type SL	0.48	2860	4020
Soy Peptone Type F*	--	4020	4250

*The peptone is insoluble.

Table 5 shows that TY is a much better fermentation medium than TYM (compare to titers in Table 4) and that Vegetable Peptone No. 1 was the best choice of 9 different non-animal peptones tested to replace Tryptone in fermentation medium for Toxin A production. It was better than Hy-Soy, which was the best in the poor TYM medium (Table 4).

Table 6. Comparison of different non-animal peptones as replacements for Tryptone in TY fermentation medium.

Peptone	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
Tryptone	0.86	2480	3900
NZ-Soy	0.96	7000	4420
NZ-Soy BL4	0.83	9000	4500
NZ-Soy BL7*	--	10000	9875
AMI-Soy*	--	2950	3500
Hy-Soy T*	--	2150	4100
SE50M	0.72	1670	3450
WGE80BT*	--	4800	3750
WGE80M	0.83	4900	3280
SE70BT*	--	2180	3650
SE70M	0.55	2775	3500
CNE50M	0.72	1800	3480
Vegetable Peptone No. 1	0.72	2750	4280
Hy-Soy	0.60	2520	3300

*The peptone is insoluble.

Table 6 shows that NZ Soy, NZ-Soy BL4, and NZ-Soy BL7 were better than Vegetable Peptone No. 1. The best was NZ-Soy BL7, an insoluble peptone. All four were better than the rest.

Table 7. Comparison of the different non-animal peptones that acted in earlier experiments as replacements for Tryptone in TY fermentation medium.

Peptone	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
Tryptone	0.91	2709	7136
Hy-Soy	0.73	4608	5312
NZ-Soy	0.97	8832	11136
NZ-Soy BL7*	--	8704	12032
SE50M	0.88	2091	8128
CNE50M	0.72	3200	5216
WGE80M	0.77	7808	9472
Plant Peptone E1	0.66	2645	3531
Vegetable Peptone No. 1	0.75	1584	12544
Soy Peptone Type II	0.66	4960	5888
Soy Peptone Type AC	0.72	3477	6496
Soy Peptone Type AB	0.74	4384	5024
Soy Peptone Type SL	0.59	3627	4672
NZ-Soy BL4	0.81	8032	9472

*The peptone is insoluble.

Table 7 shows that at 5 days Vegetable Peptone No. 1 and NZ-Soy BL7 were the best peptones for Toxin A production. NZ-Soy was almost as good and somewhat better than NZ-Soy BL4. At 3 days, NZ-Soy BL4 was best, and NZ-Soy and NZ-Soy BL7 were almost as good, but Vegetable Peptone No. 1 was poor. We have thus identified 4 good replacements for Tryptone. They are NZ-Soy BL7 (insoluble), NZ-Soy BL4, NZ-Soy, and Vegetable Peptone No. 1.

Section 4: To determine whether non-animal peptone products can replace Tryptone in seed medium for cell growth and Toxin A production. (In sections 4-9, the working cell bank stock culture is prepared in a Tryptone-containing medium).

Table 8. Comparison of the following seed media: TYM medium (control), TY medium, and VPY medium (Vegetable Peptone No. 1 replacing Tryptone in TY medium)*.

Seed Media	Cell growth ($OD_{540\ nm}$ at 24 hours) 2^{nd} stage seed	Fermentation	Toxin A production (ng/ml) 3 days	5 days
TYM	1.02	0.73	2080	4280
TY	1.02	0.93	500	1800
VPY	0.98	0.94	400	1100

*Fermentation was done in medium containing Vegetable Peptone No. 1 as a replacement for Tryptone in TY medium.

Table 8 shows that Toxin A production was lower when TY medium or TY containing Vegetable Peptone No. 1 as a Tryptone replacement was used as seed media. TYM was a much better seed medium for Toxin A production, despite the observation that growth was poorer in fermentation medium than with the other two seed media. TYM contains mannitol and glycerol. Thus, carbon sources in seed medium facilitate development of a good inoculum.

Table 9. Comparison of SYM seed medium (NZ-Soy BL4 as Tryptone replacement in TYM seed medium) with TYM seed medium*.

Seed Media	Cell growth ($OD_{540\ nm}$ at 24 hours) 2^{nd} stage seed	Fermentation	Toxin A production (ng/ml) 3 day	5 day
TYM	1.09	0.81	8032	9472
SYM	0.99	0.96	3712	5024

*Fermentation was done in NZ-Soy BL4 fermentation medium.

Table 9 shows that Toxin A production was lower in the NZ-Soy BL4 fermentation medium when NZ-Soy BL4 was used as a Tryptone replacement in the TYM seed medium. Although not as good as Tryptone, NZ-Soy BL4 led to about half the toxin production of the Tryptone seed medium.

Table 10. Comparison of different non-animal peptones as Tryptone replacements in TYM seed medium*.

Seed Media	Cell growth ($OD_{540\ nm}$ at 24 hours) 2nd stage seed	Fermentation	Toxin A production (ng/ml) 3 days	5 days
Tryptone	1.10	0.81	8320	10112
Hy-Soy	0.90	0.98	2192	2816
NZ-Soy	1.04	0.97	2720	3264
NZ-Soy BL7**	--	1.00	2080	2848
SE50M	0.97	0.99	2837	3072
WGE80M	0.89	0.99	2048	2816
Plant Peptone E1	0.67	0.99	3221	3627
Vegetable Peptone No. 1	0.75	0.96	1964	3029
Soy Peptone Type II	0.87	0.95	2059	3552
Soy Peptone Type AC	1.00	0.97	3125	3755
Soy Peptone Type AB	0.94	0.95	3605	3605
NZ-Soy BL4	0.95	0.99	3540	4181

*Fermentation was done in NZ-Soy BL4 fermentation medium.
**The peptone is insoluble.

Table 10 shows that the best non-animal peptone for seed medium was NZ-Soy BL-4. Plant Peptone E1, Soy Peptone Type AC, and Soy Peptone Type AB were next best for replacing the Tryptone in the TYM seed medium. However, Toxin A production was about 2.4 times higher when the seed medium contained Tryptone rather than NZ-Soy BL-4, both at 3 and 5 days.

Table 11. Comparison of different non-animal peptones as Tryptone replacements in TYM seed medium (pH 7.5)*.

Seed Media	Cell growth ($OD_{540\ nm}$ at 24 hours) 2nd stage seed	Fermentation	Toxin A production (ng/ml) 3 days	5 days
Tryptone	1.11	0.79	6818	8416
AMI Soy**	--	0.99	2645	3541
Hy-SoyT**	--	0.97	2140	4235
SE50MK	0.96	1.01	2507	2976
WGE80BT**	--	0.96	2564	3211
SE70BT**	--	0.91	2720	4960
SE70M**	--	0.91	2837	4085
CNE50M	0.92	0.93	1984	2731
Soy Peptone A1	0.95	0.93	2456	3712
Soy Peptone A2	1.03	0.96	3285	4672
Soy Peptone A3	1.04	0.94	3573	4704
Plant Peptone ET1**	--	0.94	2667	3529
Wheat Peptone E1	0.87	0.95	2816	3317
NZ-Soy BL4**	--	0.94	3467	3808

*Fermentation was done in NZ-Soy BL4 fermentation medium.
**The peptone is insoluble at pH 7.5.

Table 11 shows that the better non-animal peptones for seed media were Soy Peptone A3 (soluble), Soy Peptone A2 (soluble), SE70BT (insoluble), SE70M (insoluble), and HY-Soy T (insoluble) as replacements for the Tryptone in the TYM seed medium. However, Toxin A production was still much higher when the seed medium contained Tryptone (at both 3 and 5 days). The best non-animal peptone for seed medium considering both 3 and 5 days of fermentation was Soy Peptone A3, which yielded 52% of the Tryptone titer at 3 days and 56% at 5 days.

Section 5: To determine the effect of initial pH of seed medium on cell growth and Toxin A production.

Table 12. Comparison of initial pH of NZ-Soy BL4 seed medium for cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

Test No.	pH of seed medium	Cell growth ($OD_{540\ nm}$ at 24 hours) 2^{nd} stage seed	Fermentation	Toxin A production (ng/ml) 3 days	5 days
I	6.8	0.95	0.99	3540	4181
	7.5	--*	0.93	5152	5120
	8.5	--*	0.95	3349	4395
II	6.8	0.98	0.92	2160	3050
	7.5	--*	0.94	3488	3808

*The peptone was insoluble at these higher pH values.

Table 12 shows that cell growth was similar in the fermentation medium, but Toxin A production increased when the pH of the seed medium was raised from 6.8 to 7.5. Toxin A production decreased when the pH of the seed medium was further increased to 8.5. From this experiment on, the initial pH of the seed media was 7.5 for control.

Table 13. Comparison of initial pH of Soy Peptone A3 seed medium for cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

pH of seed medium	Cell growth ($OD_{540\ nm}$ at 24 hours) 2^{nd} stage seed	Fermentation	Toxin A production (ng/ml) 3 days	5 days
7.5	1.10	0.77	8480	10752
8.5	1.10	0.73	9856	11904

Table 13 shows a slightly positive effect on Toxin A production at both 3 and 5 days when pH of Soy Peptone A3 seed medium was increased from 7.5 to 8.5.

Section 6: To determine the effect of different concentrations of nitrogen source in seed media on cell growth and Toxin A production

Table 14. Comparison of different concentrations of NZ-Soy BL4 in seed medium for cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

NZ-Soy BL4 in seed medium (g/L)	Cell growth ($OD_{540\ nm}$ at 24 hours)		Toxin A production (ng/ml)	
	2nd stage seed	Fermentation	3 days	5 days
24 (control)	0.95	0.99	3540	4181
48	0.95	0.93	3904	3755
96	1.00	0.94	3851	4704

Table 14 shows that cell growth and Toxin A production were not markedly affected when NZ-Soy BL4 in seed medium was increased from 24 g/L up to 96 g/L.

Section 7: To determine the effect of inoculum concentration used for fermentation stage on cell growth and Toxin A production

Table 15. Comparison of inoculum concentration in fermentation stage on cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

Inoculum concentration (%)	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml)	
		3 days	5 days
0.5 (control)	0.76	12288	11008
1.0	0.75	8576	11904
5.0	0.77	8896	12928

Table 15 shows a negative effect on Toxin A production at 3 days when increased inoculum volume was used for fermentation. At 5 days, there was a slight stimulation. It would appear that 0.5% is satisfactory as an inoculum concentration for fermentation.

Section 8: To determine the effect of reduced iron powder and soluble iron compounds added to second stage seed media on cell growth and Toxin A production.

Table 16. Effect of reduced iron powder (0.5 g/L) in the second stage seed medium on cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

Seed media	Cell growth ($OD_{540\ nm}$ at 24 hours)		Toxin A production (ng/ml)	
	2nd stage seed	Fermentation	3 days	5 days
Test I				
Soy Peptone A3	1.03	0.76	12288	11008
Soy Peptone A3+iron pdr.	--*	0.72	11264	15744
Test II				
Soy Peptone A3	1.10	0.77	8480	10752
Soy Peptone A3+iron pdr.	--*	0.71	11008	14208
NZ-Soy BL4	--**	0.90	2548	3703
NZ-Soy BL4+iron pdr.	--*	0.87	3776	5568

*The iron powder is insoluble.
**The peptone was insoluble at pH 7.5.

Table 16 shows that Toxin A production was markedly increased at 5 days when 0.5 g/L reduced iron powder was added into the second stage seed medium. In all cases but one, it also increased production at 3 days.

Table 17. Comparison of different iron compounds in second stage Soy Peptone A3 seed medium on cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

Iron compounds in second stage seed medium (g/L)	Cell growth ($OD_{540\ nm}$ at 24 hours)		Toxin A production (ng/ml)	
	2nd stage seed	Fermentation	3 days	5 days
None	1.10	0.77	8480	10752
Reduced iron powder (0.5)*	--	0.71	11008	14208
$FeSO_4 \cdot 7H_2O$ (0.04)	1.05	0.75	8704	12160
Ferrous gluconate (0.2)	1.02	0.75	9728	13184

*The iron powder is insoluble.

Table 17 confirms that Toxin A production is increased both at 3 and 5 days when 0.5 g/L reduced iron powder is added into the second stage seed medium. Toxin A production was increased at 5 days when 40 mg/L $FeSO_4$ and 200 mg/L ferrous gluconate were added into the second stage seed medium, but $FeSO_4$ was not stimulatory at 3 days.

Iron powder is thus a useful additive to seed media containing vegetable peptones. If the insolubility is a problem, ferrous gluconate can be used.

Section 9: To determine the effect of vitamin B_{12} added to second stage seed medium on cell growth and Toxin A production.

Table 18. Effect of adding vitamin B_{12} in Soy Peptone A3 second stage seed medium on cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

Vitamin B_{12} (μg/L)	Cell growth ($OD_{540\ nm}$ at 24 hours)		Toxin A production (ng/ml)	
	2^{nd} stage seed	Fermentation	3 days	5 days
seed medium without mannitol and glycerol				
0	0.86	0.93	2667	3168
0.5	0.92	0.92	3349	3913
5	0.92	0.91	3733	3861
50	0.97	0.91	3275	3989
Seed medium with mannitol and glycerol				
0	1.03	0.76	12288	11008
0.5	1.08	0.76	7904	11392
5	1.10	0.80	7968	9632
50	1.08	0.78	7616	11008

Table 18 shows that Toxin A production slightly increased when Vitamin B_{12} was added into the Soy peptone A3 second stage seed medium that did not contain mannitol and glycerol. However, when seed medium contained mannitol and glycerol, Vitamin B_{12} had a negative effect on Toxin A production at 3 days but not at 5 days.

Section 10: To determine whether a non-animal peptone can replace Tryptone in medium for preparation of working cell bank (WCB) stock cultures of *C. difficile* VPI10463.

Table 19. Comparison of vegetable peptones in media for preparation of stock cultures and seed media on cell growth and Toxin A production in NZ-Soy BL4 fermentation medium.

WCB peptone	Seed media	Cell growth ($OD_{540\,nm}$ at 24 hours)		Toxin A production (ng/ml)	
		2nd stage seed	Fermentation	3 days	5 days
Test I					
Tryptone	TYM	1.10	0.86	4576	5568
Soy Peptone A3	SYM**	0.86	0.93	12288	11008
Test II					
Tryptone	TYM	1.13	0.73	5408	6499
Soy Peptone A3	SYM	1.10	0.77	8480	10752
NZ-Soy BL4*	ZYM***	--	0.90	2548	3703

*The peptone is insoluble at pH 7.5.
**Soy Peptone A3 replaced Tryptone in TYM.
***NZ-Soy BL4 replaced Tryptone in TYM.

Table 19 shows that the medium for preparing working cell bank stock culture WCB8.9.0-SPA3 containing Soy Peptone A3 was excellent. Using it, Toxin A production was much higher with Soy Peptone A3 as the Tryptone replacement in TYM seed medium for first stage seed culture and second stage seed culture than the control situation using the old cell bank stock culture prepared with Tryptone in TYM. Toxin A production was much lower with NZ-Soy BL4 than with Tryptone or Soy Peptone A3 in working cell bank stock culture medium.

The results show that vegetable peptones can be used in all 4 stages of the process, i.e., working cell stock culture preparation medium, first stage seed medium, second stage seed medium, and fermentation medium.

Section 11: To determine the effect of raising the initial pH of fermentation medium on growth and Toxin A production

Table 20. Effect of pH of fermentation media on cell growth and Toxin A production with Soy Peptone A3 seed medium.

Fermentation Media	Initial pH	Cell growth ($OD_{540\ nm}$ at 24 hours)	Toxin A production (ng/ml) 3 days	5 days
Soy Peptone A3	6.8	0.69	1024	5504
Soy Peptone A3	7.5	0.69	5024	6240
NZ-Soy BL4	6.8	0.77	8480	10752
NZ-Soy BL4	7.5	--*	6656	9056

*The peptone is insoluble at pH 7.5.

Table 20 shows that increasing pH of NZ-Soy BL4 fermentation medium from 6.8 to 7.5 decreased Toxin A production. Increasing the pH of Soy Peptone A3 fermentation medium from 6.8 to 7.5 increased Toxin A production. Since NZ-Soy BL4 is a better Tryptone replacement in fermentation medium than Soy Peptone A3, NZ-Soy BL4 fermentation medium should be used in the future and the pH should not be increased to 7.5.

What is claimed is: